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ORIGINAL ARTICLE

IL-6 and IL-10 in post-transplant lymphoproliferative disorders development and maintenance: a longitudinal study of cytokine plasma levels and T-cell subsets in 38 patients undergoing treatment

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Summary

IL-6 and IL-10 have previously been implicated in the pathogenesis of post-transplant lymphoproliferative disorders (PTLD) and, like peripheral lymphocyte populations, are markers of immune status that are amenable to study *in vivo*. Thus, we analyzed cytokine plasma levels as well as lymphocyte subsets in a longitudinal analysis of 38 adult transplant recipients undergoing treatment for PTLD. Pretherapeutically, we found significantly elevated IL-6 (13.8 pg/ml) and IL-10 plasma levels (54.7 pg/ml) – in the case of IL-10, even higher in treatment nonresponders than in responders (116 vs. 14 pg/ml). Over time, however, IL-10 levels did not correlate with the course of disease, whereas those of IL-6 did, falling in responders and rising in nonresponders. These findings were independent of histological EBV-status, treatment type, and total peripheral T-cell counts, which were significantly reduced in patients with PTLD. Our observations support the idea that although IL-10 is important for creating a permissive environment for post-transplant lymphoma development, IL-6 is associated with PTLD proliferation. The analysis of lymphocyte subsets further identified HLA-DR+ CD8+ lymphocyte numbers as significantly different in non-PTLD controls (33%), treatment responders (44%) and nonresponders (70%). Although the specificity of these cells is unclear, their increase might correlate with the impaired tumor-specific cytotoxic-T-lymphocyte (CTL)-response in PTLD.

Introduction

Post-transplantation lymphoproliferative disorder (PTLD) represents one of the most frequent malignancies after solid organ transplantation. It has been associated with all of the immunosuppressive agents currently in use [1,2], but the highest incidences of PTLD have been observed after treatment with T-cell depleting antibodies [3]. Reconstitution of the immune response through a reduction of immunosuppressant treatment can lead to

remission of PTLD, particularly in early lesion or polymorphic PTLD [4,5]. Thus, the key permissive factor for the development of PTLD is an impaired immune response. In addition, a considerable number of reports have documented the association of PTLD with EBV infection, in particular with primary EBV infection post-transplant [2,6]. Although only about 50–60% of PTLDs are currently regarded as EBV-associated on the basis of histology [7–10], EBV is still considered the major biologic trigger of malignant transformation in this entity [11,12].

The underlying immune regulatory mechanisms are poorly understood. Aspects that can be studied *in vivo* in PTLD patients over time are the plasma levels of key regulatory cytokines and peripheral lymphocyte subpopulations. A number of cytokines have been shown to be involved in the regulation of B-cell proliferation and immunity to intracellular pathogens and malignant cells [13]. Although IL-6 and IL-10 modulate B-cell activation and/or proliferation [13–17], TNF-alpha contributes to viral defense via its pro-inflammatory activity [13,18]. Polymorphisms of cytokine coding and promoter sequences have been shown to influence plasma cytokine levels [19]. We have recently described polymorphisms in the TGF-beta and IL-10 promoters that are associated with susceptibility to PTLD [20]. Others have postulated plasma IL-10 as a possible marker for the early detection of PTLD in transplant recipients [16,21–23]. The corresponding data for IL-6 is lacking, but IL-6 neutralizing monoclonal antibodies have been successfully used therapeutically in polymorphic PTLD [17]. In addition, EBV interferes with the host cytokine milieu by producing a viral IL-10 homolog that has been suggested to constitutively activate an autocrine signaling pathway involving Jak/STAT [24]. This suggests distinctive roles for IL-6 and IL-10 in the pathogenesis of PTLD. Available studies of cytokine plasma levels in PTLD published so far are limited by their small sample size, retrospective format, and single-point-analysis. To improve our understanding of the etiology of PTLD, we embarked on a prospective, longitudinal study of IL-6, IL-10, and TNF-alpha plasma levels in adult solid organ transplant recipients undergoing treatment for PTLD. In addition, we monitored lymphocyte counts including T-cell subpopulations and compared pretreatment values of cytokine plasma levels and T-cell numbers with appropriate transplant controls.

Materials and methods

Patient inclusion and sample collection

This study was conducted using pooled data from the PTLD-1 trial and single patient data from first-line therapy in adult transplant recipients prospectively reported to the German PTLD registry (PTLD D2006–2012). The PTLD-1 trial commenced in 2003, and is a prospective phase II trial using sequential treatment comprising four courses of rituximab followed by four courses of CHOP chemotherapy as a first-line treatment in PTLD unresponsive to immunosuppression reduction (IR) [25]. This trial had a major amendment in 2006 introducing risk-stratified sequential treatment (RSST) [26]. Under the RSST-approach, patients with a complete response (CR) after four courses of rituximab monotherapy were regarded as ''low risk'', whereas patients with a partial response (PR), stable disease (SD), or progressive disease (PD) were regarded as ''intermediate/high risk''. Low risk patients were treated with four further courses of rituximab monotherapy (once every 3 weeks). High-risk patients subsequently received four courses of R-CHOP. Finally, the PTLD D2006–2012 trial is a prospective national registry for rare PTLD subtypes and relapsed PTLD using different treatment protocols for distinct subtypes. The responsible local ethical committees approved these trials in December 2002 and June 2007, and all patients gave written informed consent according to the Declaration of Helsinki. Between 11/2005 and 05/2009, longitudinal sampling for cytokine plasma level measurements was performed in a subset of 38 newly diagnosed PTLD patients consecutively enrolled and followed up at the Department of Hematology. Blood samples were taken before the start of a treatment cycle or at follow-up appointments, and all cytokine measurements were performed on the day of collection.

Diagnosis, treatment, response assessment and follow-up

The diagnosis of PTLD was based on an examination of histologic material obtained by open biopsy or core needle biopsy. Diagnostic tissue samples were reviewed by a single expert pathologist. To detect lymphoproliferative sites, all patients diagnosed with PTLD underwent a physical examination, blood tests [full blood count, serum electrolytes, liver function tests, lactate dehydrogenase (LDH, upper limit 248 units/l)], a bone marrow biopsy, and computed tomography (CT) scans of the neck, chest, abdomen, and pelvis.

Although there were inter-individual differences in patient treatment depending on whether the patient was included in the PTLD-1 trial or in the PTLD registry, assessment of treatment response was routinely based on follow-up CT imaging of all involved regions, and was performed both during and 4 weeks after completion of treatment. Follow-up assessment was initially conducted at a minimum of 6, 12, 18, and 24 months after completion of treatment and then yearly. Remission was defined in accordance with WHO criteria. However, for the correlation of cytokine plasma levels to treatment response,

CR and PR patients were grouped together as ''responders'', whereas SD and PD patients were grouped together as ''nonresponders''.

Cytokine measurements

Cytokine measurements were performed at the Institute for Medical Immunology at Charité Campus Mitte using the Immulite® automated immunoassay system (DPC Biermann, Bad Nauheim, Germany) according to the manufacturer's specifications. The lowest level of detection was 2 pg/ml for IL-6, 5 pg/ml for IL-10, and 1.7 pg/ml for TNFa. Heparinised plasma was used for the IL-10 and TNFa measurements, whereas IL-6 was quantified in EDTA-plasma.

Flow cytometry

Cell phenotype was analyzed by staining with fluorochrome-conjugated monoclonal antibodies for the surface markers CD3, CD8, CD4, HLA-DR, CD57, CD28 and CD11a, CD19 and CD16 (BD Biosciences, San Jose, CA, USA) to determine T cells, B cells and natural killer cells, respectively. Four-color flow cytometry was performed using FACSCalibur™ and CELLQuest™ Software (BD Biosciences, Franklin Lakes, NJ, USA).

Identification of controls

To identify the distribution of cytokine plasma levels in non-PTLD transplant recipients, a control group similar to the study group was formed by an automated, computerized process. In total, 42 stable transplant patients from the transplant outpatient departments of the Charité – Universitätsmedizin Berlin (Campus Virchow-Klinikum) were randomly selected based on frequency matching. The matching criteria were age, sex, graft, time from transplantation to diagnosis/study inclusion, and type of immunosuppressive treatment. The resulting baseline characteristics were comparable to the PTLD group and are presented in Table 1.

Statistical analysis

The frequencies of the patient characteristics were estimated based on the observed data. Categorical variables were compared by the chi-square test or Fisher's exact test, where applicable. Continuous variables for patient characteristics and baseline cytokine plasma levels were compared using a *t*-test for independent samples with a previous test for equality of the variances. A linear mixed-effects model (short regression analysis) [27] was used to assess the course of cytokine plasma levels over time. The response to

Table 1. Baseline characteristics of post-transplant lymphoproliferative disorders (PTLD) and control patients.

	PTLD (n = 38)	Controls (n = 42)	P
Age (mean, range) (years)	53.6 (19–82)	52.6 (31–75)	0.630†
Sex (M/F) (%)	25/13 (66)	29/13 (69)	0.814‡
Type of transplant (%)			
Kidney	15 (39)	18 (43)	0.570§
Liver	13 (34)	18 (43)	
Lung	1 (3)	1 (2)	
Combined transplant	9 (24)	5 (12)	
History of prior transplantations (%)	2/38 (5)	2/42 (5)	1.000‡
Underlying disease: autoimmune disorders* (%)	8/38 (21)	8/42 (19)	1.000‡
Time from last transplantation to study entry (mean, range) (years)	9.3 (0.2–22)	7.3 (0.2–21.5)	0.142†
IS at study entry			
CNI (%)	37/38 (97)	33/42 (79)	1.000‡
CyA (%)	9/38 (24)	15/42 (36)	0.092‡
Mean CyA plasma levels (ng/ml)	130.8	138.2	0.793†
FK (%)	28/38 (74)	18/42 (43)	0.113‡
Mean FK plasma levels (ng/ml)	7.49	7.19	0.760†
mTOR (%)	2/38 (5)	5/42 (12)	0.436‡
Mean mTOR doses (mg/day)	2.0	1.8	0.704†
MMF (%)	12/38 (32)	19/42 (45)	0.254‡
Mean MMF doses (mg/day)	1282	1566	0.148†
AZA (%)	12/38 (32)	9/42 (21)	0.322‡
Mean AZA doses (mg/day)	69	35	**0.023**†
Steroids (%)	19/38 (50)	24/42 (57)	0.654‡
Mean steroid doses (mg/day)	6.7	5.0	0.079†
Low dose steroids: ≤5 mg (%)	13/38 (34)	21/42 (50)	0.179‡
Increased steroid dosing: 5.1–10 mg (%)	4/38 (11)	2/42 (5)	0.416‡
Increased steroid dosing: >10 mg (%)	2/38 (5)	1/42 (2)	0.602‡

PTLD, post-transplant lymphoproliferative disorders.
*Following autoimmune disorders were included: autoimmune glomerulonephritis (autoimmune hepatitis, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC).
†*t*-test.
‡Fisher's exact test.
§Chi-square test.

treatment was included as a covariate together with an interaction term for the response to treatment and the time from the start of treatment. Linear mixed-effects models allow the investigation of variability between patients (heterogeneity) while simultaneously adjusting for the within-subject correlation. In the present analysis, random effects were permitted for the intercepts and for the effect of time. The inclusion of random effects implies that the change over time may vary between patients. As several potentially confounding factors might affect the cytokine plasma levels, a model reduction based on likelihood-ratio tests was performed to determine the model that provided the best fit to our observational data with the smallest set of variables. Model regression coefficients are reported together with their standard error estimates (SE). The level of significance in all cases was set at $P < 0.05$. Statistical tests were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA), SAS 9.2 (SAS Institute GmbH, Heidelberg, Germany), and R 2.92 (R Foundation for Statistical Computing, 2010).

Results

In total, 38 adult patients and 42 controls were enrolled in the study. In the PTLD group, the following organs had been transplanted: kidney (15 patients), liver (13), lung (1), and two organs (9). Mean patient age at diagnosis of PTLD was 53.6 years. Ninety seven percent of patients were on calcineurin inhibitors at diagnosis of PTLD, whereas 5% received mTOR-based immunosuppression (Table 1). PTLD usually was late PTLD occurring later than 1 year after transplantation (92%) with an aggressive monomorphic histology (89%) and an advanced stage of disease (63%) (Table 2). After diagnosis of PTLD, immunosuppression was reduced in all patients. Of the 27 patients subsequently treated according to the PTLD-1 protocol (71%), 10 patients received rituximab monotherapy and 17 received sequential treatment with rituximab and chemotherapy (either CHOP or R-CHOP). Eleven patients were enrolled in the PTLD registry: five were diagnosed with different CD20-positive B-cell PTLD subtypes, three had Hodgkin- or Hodgkin-like PTLD, one was diagnosed with angioimmunoblastic T-cell PTLD, one with plasmacytoma-like PTLD, and one with CD20-negative plasmablastic diffuse-large-B-cell (DLBCL)-PTLD. The five patients with CD20-positive B-cell PTLD initially received rituximab monotherapy and two of five also received subsequent chemotherapy (CHOP/bendamustin). Patients with Hodgkin-PTLD either received ABVD (two patients) or irradiation of involved sites (one patient). The patient with T-cell PTLD was treated with CHOP, whereas the patient with plasmacytoma-like PTLD received VAD. The patient with plasmablastic DLBCL-PTLD died from progressive disease before treatment could be initiated. After first-line treatment, 27 of 38 patients achieved CR and two of 38 patients achieved PR (responders), whereas nine patients had progressive disease (nonresponders). There were no SD patients. Eight of the nonresponding patients died from progressive disease during treatment, whereas one patient responded to subsequent salvage therapy. Two CR patients relapsed during follow-up. These last three patients' data was censored from the start of salvage treatment or from the time of the last evidence of CR, respectively. Immunosuppression at diagnosis of PTLD and after IR were not significantly different in the two response groups (Table 2). There were also no significant differences in treatment regimens and intensity: of the responders, 16/29 (55%) received CHOP-like chemotherapy and 25/29 (86%) received rituximab compared with eight of nine (89%) and five of nine (56%) of nonresponders (Table 2). The median number of applied chemotherapy cycles was four and two, respectively. One responding patient had no systemic therapy, but local irradiation of involved sites (3%), whereas one nonresponding patient had no treatment at all (11%) ($P > 0.05$ for all comparisons). As a result of early death, the median time of follow-up was 46 days in nonresponders, whereas it was 204 days in responders. With a total of 34 and 146 single-point-analyses performed in the two groups, the mean density of measurements was 1 measurement/patient every 12.2 days in nonresponders and 1 measurement/patient every 40.5 days in responders. Although none of the nonresponding patients reached the follow-up phase, the mean density of measurements of responders differed during treatment and follow with 1 measurement/patient every 42 days during the first 120 days (treatment period) and 1 measurement/patient every 111 days after day 120 (follow-up period).

Plasma IL-6, IL-10 and TNFa levels at diagnosis of PTLD

At the time of diagnosis, mean plasma levels of IL-6 were significantly elevated in patients with PTLD (13.8 vs. 3.8 pg/ml in controls, $P < 0.004$, box-plot distribution shown in Fig. 1a). There was no difference in pretherapeutic plasma IL-6 levels between responders and nonresponders ($P = 0.232$).

Pre-therapeutic IL-10 levels (Fig. 1b) were elevated in 52% of PTLD patients, but only in 10% of controls ($P < 0.001$, Fisher's exact test). Accordingly, the mean IL-10 level in PTLD patients was significantly higher than in controls (54.7 vs. 5.4 pg/ml, $P = 0.025$). There was an additional significant difference within the PTLD group with higher mean IL-10 levels in nonresponders than responders (116 vs. 14 pg/ml, $P < 0.048$).

Table 2. Characteristics of responders and nonresponders.

	Nonresponder (*N* = 9)	Responder (*N* = 29)	*P*
Underlying disease: autoimmune disorder (GN, autoimmune hepatitis, PSC, PBC) (%)	2/9 (22)	6/29 (21)	1.000†
History of prior transplantations (%)	1/9 (11)	1/29 (3)	1.000†
Time from transplantation to PTLD	1/8	2/27	0.682†
Early versus late			
Immunosuppressive treatment at diagnosis of PTLD			
CyA (%)	3/9 (33)	12/29 (41)	1.000†
Mean CyA plasma levels (ng/ml)	112	136	0.468*
FK (%)	6/9 (67)	12/29 (41)	0.260†
Mean FK plasma levels (ng/ml)	9.4	6.3	0.061*
mTOR inhibitor (%)	0/9 (0)	1/29 (3)	1.000†
MMF (%)	2/9 (22)	10/29 (34)	0.689†
Mean MMF doses (mg/day)	1500	1238	0.582*
AZA (%)	3/9 (33)	9/29 (31)	1.000†
Mean AZA doses (mg/day)	58	72	0.583*
Steroids (%)	4/9 (44)	15/29 (52)	1.000†
Low dose steroids: ≤5 mg (%)	1/9 (11)	12/29 (44)	0.126†
Increased steroid dosing: 5.1–10 mg (%)	2/9 (22)	1/29 (3)	0.134†
Increased steroid dosing: >10 mg (%)	1/9 (11)	2/29 (7)	0.134†
Mean steroid doses (mg/day)	7.5	6.7	0.750*
Immunosuppressive treatment after IR			
CyA (%)	3/9 (67)	10/29 (34)	1.000†
Mean CyA plasma levels (ng/ml)	158	160	0.980*
FK (%)	4/9 (44)	12/29 (41)	1.000†
Mean FK plasma levels (ng/ml)	5.4	5.1	0.862*
mTOR inhibitor (%)	1/9 (11)	2/29 (7)	1.000†
Mean mTOR doses (mg/day)	3.0	2.8	0.946*
MMF (%)	1/9 (11)	10/29 (34)	0.237†
Mean MMF doses (mg/day)	1000	1013	0.977*
AZA (%)	0/9 (0)	2/29 (7)	1.000†
Mean AZA doses (mg/day)	–	50	–
Steroids (%)	3/9 (33)	15/29 (52)	0.454†
Low dose steroids: ≤5 mg (%)	2/9 (22)	13/29 (45)	0.273†
Increased steroid dosing: 5.1–10 mg (%)	1/9 (11)	0/29 (0)	0.237†
Increased steroid dosing: >10 mg (%)	0/9 (0)	2/29 (7)	1.000†
Mean steroid doses (mg/day)	9.2	6.5	0.454*
Histology of PTLD			
Polymorphic B-cell PTLD (%)	2 (22)	3 (10)	0.472‡
Monomorphic B-cell PTLD (%)	6 (68)	26 (90)	
Burkitt PTLD	0	2	
DLBCL-type PTLD	5	16	
Hodgkin-PTLD	1	2	
Plasmacytoma-like PTLD	0	1	
Other B-cell PTLD	0	5	
T-cell PTLD (%)	1 (11)	0 (0)	
EBV-serology	1/8	0/29	0.237†
Primary infection/reactivation			
EBV-association of PTLD	8/1	9/20	**0.005**†
Positive/negative			
Peripheral EBV-DNA load	8/1	15/14	0.061†
Positive/negative			
Peripheral EBV-DNA load	1100 (0;70 500)	0 (0;4500)	0.117*
Median (25%-;75%-percentile) in copies/ml			
Ann Arbor stage	3/6	11/18	0.359†
Limited versus advanced disease			

Table 2. continued.

	Nonresponder (*N* = 9)	Responder (*N* = 29)	*P*
LDH Median (25%-;75%-percentile) in U/ml	339 (258;998)	209 (181;270)	**0.002***
Treatment protocol including rituximab (%)	5/9 (56)	25/29 (86)	0.071†
Treatment protocol including chemotherapy (%)	8/9 (89)	16/29 (55)	0.115†

*Mann–Whitney test.
†Fisher's exact test.
‡Chi-square test.

Differences in pretherapeutic plasma TNFa levels were less pronounced (mean 27.7 pg/ml in PTLD patients and 16.0 pg/ml in healthy transplant controls). However, this difference still reached statistical significance ($P = 0.004$). Distributional differences are illustrated in Fig. 1c. There was no difference in pretherapeutic plasma TNFa levels between responders and nonresponders ($P = 0.619$).

The course of cytokine plasma levels in responders and nonresponders to PTLD treatment

To assess differences in the plasma levels of IL-6, TNFa, and IL-10 over time in the different response groups, a linear mixed-effects model was applied. Model reduction identified a model that included random effects, time from the diagnosis of PTLD (1), response to the PTLD treatment (2), and the interaction of time and response (3) as fixed-effect parameters. This model provided the best fit to the observed data with the smallest set of variables (Table 3) and allowed to determine whether the development of the plasma level of a given cytokine over time was significantly different in the response versus the nonresponse group. In our model, this translated into a statistically significant difference for the term "Δ change over time responders" for a specific cytokine.

The subsequent solution of the three different effects models for fixed effects revealed a significant decrease of plasma IL-6 levels with time in responders to PTLD treatment, whereas nonresponders demonstrated increasing plasma IL-6 levels (Fig. 2). As shown in Table 3, there is a mean increase per day of 0.316 pg/ml (SE = 0.10, $P = 0.001$) for all patients. In the responder group, there is an additional mean decrease per day of 0.318 pg/ml (SE = 0.10, $P = 0.001$). Thus, there is a net decrease of 0.02 pg/ml/day for responders.

A significantly different time course was also identified for plasma TNFa with consistently low TNFa levels in responders, but increasing TNFa levels in nonresponders ($P < 0.001$, Table 3, Fig. 3). No significant change in plasma IL-10 levels over time was identified.

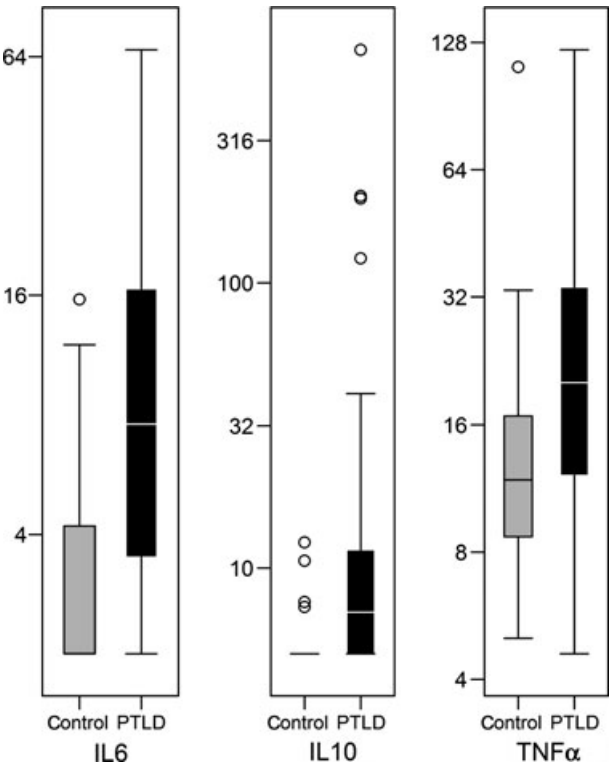

Figure 1 Cytokine levels at diagnosis of post-transplant lymphoproliferative disorders (PTLD). Patients with PTLD had significantly elevated mean IL-6 (13.8 vs. 3.8), TNFa (27.7 vs. 16.0), and IL-10 levels (54.7 vs. 5.4) at diagnosis of PTLD ($P < 0.004$ for each comparison). For a more detailed analysis, this figure illustrates the distribution of cytokine levels at diagnosis of PTLD in patients and controls using box plots. Boxes indicate the 25th, 50th, and 75th percentile of values, i.e. the middle 50% of the data sample. The remaining 50% of the sample is contained within the areas between the box and the whiskers, i.e. whiskers identify the minimal and maximal cytokine levels. Circles indicate extreme values. Extreme values were defined as being 1.5–3-fold higher than the 75th percentile or 1.5–3-fold lower than the 25th percentile, and were not used to calculate boxes and whiskers. Of note, the distribution of plasma IL-6 and IL-10 values of patients and controls were sharply separated, whereas baseline TNFa values showed a broad overlap.

Table 3. Linear mixed effect model calculation of IL-6, TNFa, and IL-10.

	Value	SE	*P*
*Cytokine plasma level – response × time (fixed effects)**			
IL-6			
Baseline (pg/ml)	**+17.760**	5.922	**0.005**
Δ baseline responders (pg/ml)	−8.928	6.503	0.172
Change over time (pg/ml/day)	**+0.316**	0.097	**0.001**
Δ change over time responders (pg/ml/day)†	**−0.318**	0.097	**0.001**
TNFa			
Baseline (pg/ml)	**+17.931**	5.441	**0.002**
Δ baseline responders (pg/ml)	+2.296	6.251	0.714
Change over time (pg/ml/day)	**+0.234**	0.062	**<0.001**
Δ change over time responders (pg/ml/day)†	**−0.236**	0.062	**<0.001**
IL-10			
Baseline (pg/ml)	**+88.360**	33.010	**0.001**
Δ baseline responders (pg/ml)	**−76.334**	38.268	**0.048**
Change over time (pg/ml/day)	−0.047	0.141	0.734
Δ change over time responders (pg/ml/day)†	+0.039	0.141	0.783
Cytokine plasma level – response × EBV-association of PTLD × time (fixed effects)			
IL-6			
Δ baseline EBV-association (pg/ml)	+11.116	17.246	0.052
Δ response EBV-associated PTLD (pg/ml)‡	−8.447	18.421	0.647
TNFa			
Δ baseline EBV-association (pg/ml)	−2.267	15.059	0.184
Δ response EBV-associated PTLD (pg/ml)‡	+10.965	17.463	0.531
IL-10			
Δ baseline EBV-association (pg/ml)	+62.437	103.930	0.549
Δ response EBV-associated PTLD (pg/ml)‡	−45.900	112.350	0.684
Cytokine plasma level – response × type of treatment × time (fixed effects)			
IL-6			
Δ change over time for rituximab treatment (pg/ml/day)§	−0.003	0.007	0.619
Δ change over time for CHOP treatment (pg/ml/day)§	−0.001	0.007	0.909
TNFa			
Δ change over time for rituximab treatment (pg/ml/day)§	+0.002	0.006	0.698
Δ change over time for CHOP treatment (pg/ml/day)§	+0.004	0.006	0.581
IL-10			
Δ change over time for rituximab treatment (pg/ml/day)§	−0.005	0.023	0.817
Δ change over time CHOP treatment (pg/ml/day)§	+0.010	0.022	0.660

PTLD, post-transplant lymphoproliferative disorders.

The statistical model allows three sources of variability for the calculation of fixed effects on cytokine plasma levels. 1: different cytokine levels at study entry (calculated baseline), 2: different effects of time, 3: random effects. For the variability between individuals a normal distribution for calculated baseline and time is assumed. The mean effect of different parameters on cytokine plasma levels is displayed together with standard error (SE) and corresponding significance level (*P*).

*As several potentially confounding factors might impact cytokine plasma levels, model reduction based on likelihood-ratio tests was performed to find the model that provided the best fit to our observational data with the smallest set of variables.

†''Δ change over time responders'' are response related effects on the cytokine plasma levels during the course of observation starting with diagnosis of PTLD to the end of follow-up. Median follow-up in this analysis is 204 days for responders and 46 days for nonresponders.

‡''Δ response EBV-associated'' identifies whether there is an interaction of EBV-association of PTLD and response to treatment.

§''Δ change over time for rituximab/CHOP treatment'' identifies whether the choice of treatment has a response-independent effect on plasma cytokine levels. ''CHOP treatment'' included patients receiving VAD and ABVD which were considered as CHOP-like.

Resulting model equations:

IL-6 plasma level = 17.760 pg/ml − 8.928 pg/ml (in patients responding to treatment) + 0.316 pg/ml/day × time (days) − 0.318 pg/ml/day (in patients responding to treatment) × time (days)

TNFa plasma level = 17.931 pg/ml + 2.296 pg/ml (in patients responding to treatment) + 0.236 pg/ml/day × time (days) − 0.234 pg/ml/day (in patients responding to treatment) × time (days)

Comment: We did not aim to predict exact cytokine plasma levels in responders and nonresponders of PTLD treatment, but tried to analyze whether elevated baseline levels normalize in responders while they further increase in nonresponders. Calculated cytokine plasma levels therefore do not necessarily match mean cytokine levels of individual patients during the course of time.

As outlined in Table 1, the frequency of histologically EBV-associated PTLD was higher in the nonresponse group ($P = 0.005$, Fisher's exact test), whereas neither median EBV-DNA load nor the frequency of positive EBV-DNA PCR were significantly different. To exclude any impact of EBV-association on the cytokine data, extended sets of linear mixed-effects models were applied including the EBV-association of PTLD as an additional fixed-effect parameter. We also tested for an interaction of EBV-association and response. Importantly, all these models failed to identify a significant effect of EBV on the cytokine data, although they confirmed the major impact of treatment response on the course of IL-6 over time ($P = 0.001$ for the effect of response, $P = 0.052$ for an impact of EBV, $P = 0.647$ for a possible interaction of EBV and response).

An important covariate influencing cytokine levels in this setting is the type of treatment. We therefore analyzed in detail whether there was an imbalance in the type and amount of immunosuppression or the use of rituximab and CHOP in the two response groups. Although immunosuppression was very well balanced (Table 2), there were some differences in the use of rituximab and CHOP in responders and nonresponders. Although these differences did not reach statistical significance, we applied extended linear mixed-effects models with treatment modality as an additional fixed-effects parameter to exclude any effect of treatment bias on the timecourses of cytokines (see Table 3). Importantly, these models confirmed the major impact of treatment response on the course of IL-6 ($P = 0.0015$) and TNFa ($P < 0.001$) over time. Similar to the initial models, the additional mean decrease of IL-6 and TNFa was calculated with 0.314 and 0.240 pg/ml/day in responders when compared with nonresponders. In contrast, neither rituximab nor CHOP treatment had a significant impact on the timecourse of IL-6 and TNFa ($P = 0.619$ and $P = 0.909$). The calculated additional mean change was 0.003 and 0.001 pg/ml/day for IL-6 and 0.003 and 0.004 pg/ml/day for TNFa.

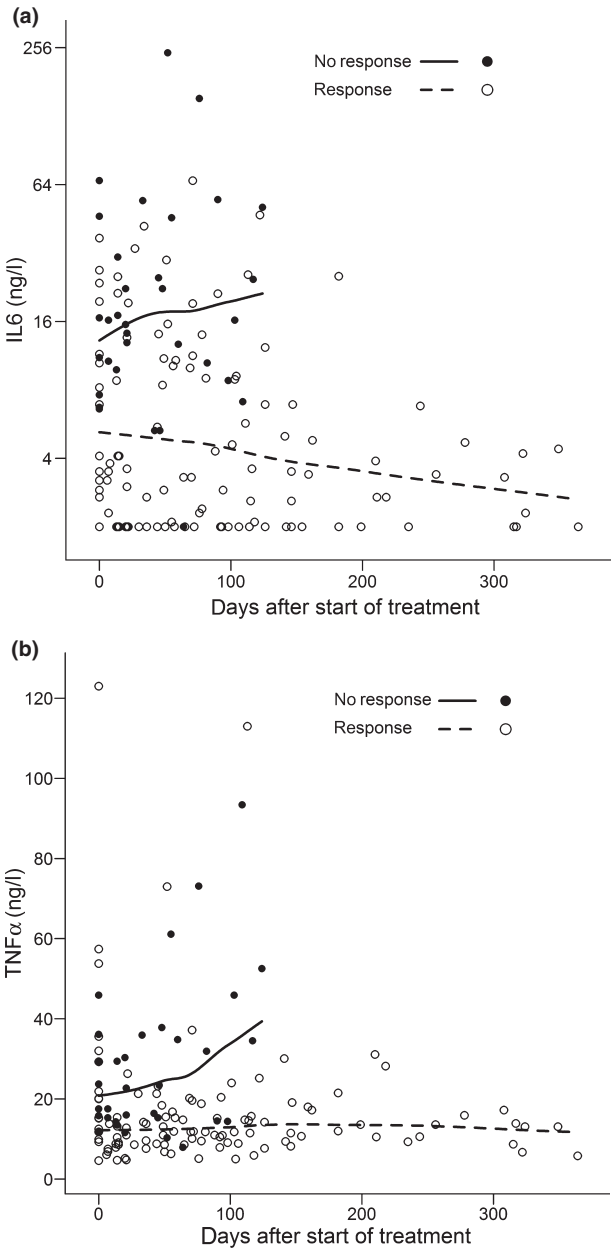

Figure 2 Plasma IL-6 (a) and TNFa (b) levels in patients undergoing post-transplant lymphoproliferative disorders (PTLD) treatment. Open circles indicate patients responding to treatment, closed circles indicate nonresponding patients. Respective lines indicate the change of interleukin levels over time for the two response groups as determined by the calculation of local means. (a) Although IL-6 decreased in patients responding to therapy, IL-6 levels increased in progressive disease. This time-dependent difference was significant (Table 3, IL-6: Δ change over time responders, $P = 0.001$). The differences in baseline IL-6 levels (Table 3, IL-6: Δ baseline responders) did not reach statistical significance ($P = 0.17$). (b) TNFa plasma levels remained stable in patients responding to therapy, whereas nonresponding patients demonstrated a significant increase in TNFa plasma levels over time (Table 3, TNFa: change over time, $P < 0.001$). There was no significant difference in baseline TNFa levels (Table 3, TNFa: Δ baseline responders, $P = 0.714$). The median time of follow-up is significantly shorter in nonresponders (46 days) compared with responders (204 days). This is a result of the higher mortality in patients not responding to 1st-line treatment.

T-cell counts and lymphocyte T-cell subsets at diagnosis of PTLD

At time of diagnosis, patients with PTLD showed significantly lower peripheral T-cell counts with a mean of 872

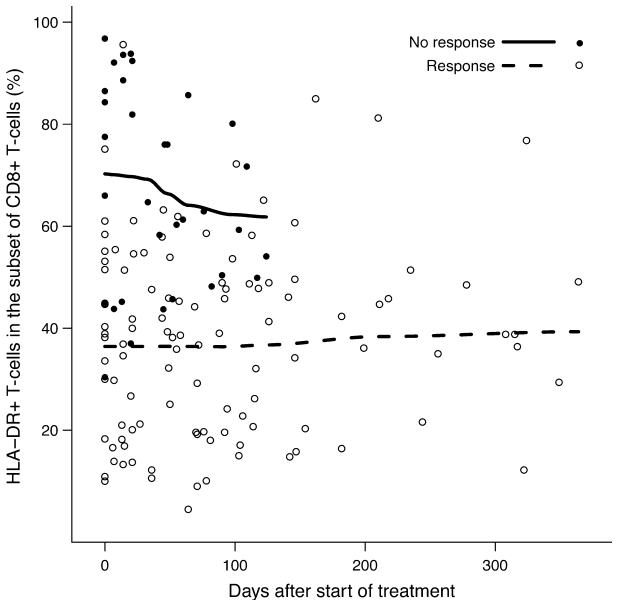

Figure 3 The frequency of activated cytotoxic T-cell in patients undergoing post-transplant lymphoproliferative disorders (PTLD) treatment. Open circles indicate patients responding to treatment, closed circles indicate nonresponding patients. Respective lines indicate the change of activated cytotoxic T-cell frequencies over time for the two response groups as determined by the calculation of local means. Pretherapeutic HLA-DR+CD8+ T-cell frequencies are significantly higher in patients with PTLD when compared with transplant controls (52.0% vs. 33.0%, $P = 0.003$). Patients not responding to treatment show even higher levels (70%) when compared with responders (44%, $P = 0.008$). This alteration in the frequency of activated cytotoxic T cells remains stable with treatment of PTLD (Table 4: change over time nonresponder: −0.163% per day, change over time responder: −0.01% per day).

Table 4. Linear mixed effect model calculation of CD19 and CD3 lymphocyte counts.

Fixed effects			
Cell counts – response × time			
	Value	SE	*P*
Total leukocytes			
Baseline (/nl)	8.102	1.106	**<0.001**
Δ baseline responders (/nl)	−0.867	1.269	0.495
Change over time (/nl/day)	+0.004	0.011	0.726
Δ change over time responders (/nl/day)	−0.004	0.011	0.723
CD3+ cells			
Baseline (/nl)	0.935	0.150	**<0.001**
Δ baseline responders (/nl)	−0.190	0.172	0.272
Change over time (/nl/day)	−0.006	0.002	**0.001**
Δ change over time responders (/nl/day)	+0.006	0.002	**0.001**
CD3+4+ cells			
Baseline (/nl)	0.2882	0.059	**<0.001**
Δ baseline responders (/nl)	+0.036	0.066	0.587
Change over time (/nl/day)	−0.002	0.001	0.053
Δ change over time responders (pg/ml/day)	+0.002	0.001	**0.049**
CD3+8+ cells			
Baseline (/nl)	0.578	0.110	**<0.001**
Δ baseline responders (/nl)	−0.198	0.127	0.121
Change over time (/nl)	−0.004	0.001	**<0.001**
Δ change over time responders (/nl/day)	+0.004	0.001	**<0.001**
HLA-DR+ cells in the subset of CD8+ cells			
Baseline (%)	74.088	6.599	**<0.001**
Δ baseline responders (%)	−32.481	7.544	**<0.001**
Change over time (%)	−0.163	0.059	**0.007**
Δ change over time responders (%)	+0.161	0.059	**0.007**

No significant changes were found in the analysis of CD19+, CD16+ CD57+, CD28+, and CD11a+ CD8+/CD8− T-cell subsets nor in the analysis of CD4+CD8+ and CD4−CD8− T-cell subsets.

CD3-positive cells/μl compared with 1182 CD3-positive cells/μl in controls ($P = 0.040$). Median T-cell counts were 830/μl and 1045/μl, respectively ($P = 0.045$). More specifically, mean CD4+ T cells counts were reduced (356/μl vs. 546/μl, $P = 0.010$) while there was no significant difference in mean CD8+ T-cell counts ($P = 0.344$). Within the different T-cell subsets, the most evident difference were mean higher frequencies of both HLA-DR+CD8+ and HLA-DR+CD4+ T cells [52% and 25% vs. 33% and 16% in controls ($P = 0.003/0.004$)]. We also found slightly higher frequencies of CD11a+ CD8+ T cells (87% vs. 77%, $P = 0.007$). The analysis of B-cell, NK-cell, CD57+ CD8+, CD57+ CD4+, CD28+ CD8+, CD28+ CD4+, and CD11a+ CD4+ T-cell counts showed no significant differences ($P > 0.05$ for all comparisons). At diagnosis of PTLD there was an additional significant difference in mean frequencies of HLA-DR+ cytotoxic T cells in nonresponders compared with responders (70% vs. 44%, $P = 0.008$), whereas total CD3+, CD4+ and CD8+ T-cell counts showed no significant differences ($P > 0.05$ for all comparisons).

Changes in T-cell subpopulations over time in responders and nonresponders to PTLD treatment

To exclude the possibility that differences in cytokine plasma levels were merely a reflection of differences in lymphocyte numbers or subpopulations, we applied linear mixed-effects models to peripheral lymphocyte populations as well (Table 4). Although we were unable to detect any consistent change in the total leukocyte count over time in the different response groups, both, CD3+ CD4+ and CD3+ CD8+ T-cell counts significantly decreased over time in nonresponders compared with responders ($P = 0.049$ and $P < 0.001$, respectively). The observed difference in the proportion of HLA-DR+ cytotoxic T cells in nonresponders (70%) compared with responders (44%) was significant throughout treatment and follow-up

($P < 0.001$, Fig. 3). For all other B-, T-, and NK-cell lymphocyte subpopulations, no significant changes over time or with respect to response were found. Again, after the inclusion of EBV-association into the fixed-effects model, the effect of response on the proportion of HLA-DR+ cytotoxic T cells over time remained significant ($P = 0.011$), whereas there was no effect of EBV-association on this subset ($P = 0.107$) and no obvious interaction between EBV-association and response ($P = 0.216$).

Discussion

In this study, we could demonstrate significantly elevated pretherapeutic plasma levels of IL-6 and IL-10 in PTLD compared with transplant controls, confirming earlier data [14,16,21–23]. Although there was no difference in baseline IL-6 between responders and nonresponders, baseline IL-10 levels were significantly elevated in nonresponders compared with responders. Using a mathematical model, we could show for the first time that in PTLD patients, the plasma levels of IL-6 and TNFa rise significantly over time in nonresponders, whereas in responders, TNFa levels remain in the normal range and increased baseline IL-6 levels fall. There was no correlation between the timecourse of IL-10 plasma levels and response. Regarding potential confounding factors, we considered in particular EBV-status, peripheral lymphocyte counts, and a pro-inflammatory role of PTLD treatment. First, we could not demonstrate a significant effect of histological EBV-status on the plasma levels of IL-6, IL-10, and TNFa. Secondly, baseline CD3+, CD4+, and CD3+ CD8+ T-cell counts were similar in responders and nonresponders at diagnosis of PTLD, and we observed rising IL-6 and TNFa levels together with simultaneously decreasing CD3+, CD4+, and CD3+ CD8+ T-cell counts in nonresponders. Thirdly, although treatment might be a cause of inflammation, neither treatment strategies nor treatment intensity significantly differed between the two response groups. Moreover, extending the mathematical model to include treatment modality as a parameter did not identify a significant effect on cytokine levels. Therefore, we believe that the observed differences in cytokine plasma levels are unlikely because of EBV, peripheral T cells numbers or treatment. However, the high frequency of monomorphic, late, EBV-negative PTLD limits the generalization of the findings to all PTLD.

The elevated IL-6 plasma levels at diagnosis of PTLD in both responders and nonresponders rise even further in nonresponders parallel to uncontrolled tumor proliferation, whereas they fall in responders achieving tumor control. Thus, IL-6 behaves like a proliferation-associated cytokine. Even though the source of the plasma IL-6 in PTLD remains unclear, the lack of a positive correlation with peripheral lymphocyte levels and EBV-status might point to secretion of IL-6 from proliferating tumor cells or activated tumor-surrounding T cells. As it is often the case with observational data, cause and effect, in this case whether IL-6 is the cause of or a consequence of malignant proliferation, are difficult to differentiate. However, one clinical study has reported that the *in vivo* neutralization of IL-6 by monoclonal anti-IL-6 antibodies in PTLD induced a clinical response [17]. Of twelve patients treated with this antibody, eight achieved disease remission (five CR, three PR). A possible mechanistic basis for the success of anti-IL-6 treatment is outlined by data from HIV-related DLBCL. First, it has been demonstrated that many tumor cells express IL-6R, rendering them responsive to paracrine stimulation by IL-6 which the tumor clone itself generally fails to release [28]. Secondly, a further study has indicated that the sensitivity of immunoblastic HIV-related DLBCL to TGF-beta1-mediated growth inhibition may be overcome through the stimulation of proliferative and anti-apoptotic signals by IL-6, particularly the rapid activation of STAT3 [29]. Taken together, there is now preclinical data from HIV-related lymphoma as well as clinical data from both longitudinal observation and intervention with anti-IL-6 antibodies in PTLD to suggest an important role for IL-6 in both development and maintenance of malignant lymphocyte proliferation in a background of immunosuppression.

Unlike IL-6, baseline IL-10 plasma levels were not only elevated in PTLD compared with controls, but were also significantly higher in responders than nonresponders. However, they did not correlate with the course of disease. IL-10 levels play a key role in Th1/Th2 balance [30], which is thought to be critically important for efficient antitumor immunity [31]. Polymorphisms in IL-10 have been shown to be associated with susceptibility to EBV-associated PTLD possibly resulting from a change in the inflammatory response to EBV [20]. In contrast to IL-6, this is consistent with a role of IL-10 in creating a permissive situation for autonomic B-cell proliferation in the immunosupressed rather than in maintaining proliferating PTLD.

Tumor necrosis factor-a plasma levels increased in nonresponders, whereas pretherapeutic plasma TNFa levels were not significantly elevated in PTLD. Thus, in contrast to IL-6 and IL-10, levels of TNFa seem unrelated to the presence of PTLD, and might be interpreted as the result of an inflammatory response to uncontrolled, progressive disease.

In our analysis of lymphocyte populations, we observed lower CD3+ and CD4+ counts in patients with PTLD compared with controls, in keeping with the notion that patients with PTLD are biologically over-immunosuppressed even though controls in this study were matched for comparable immunosuppressant treatment. This is in

line with previous observations on CD4+ cells [32] and reinforces the rationale for reduction of immunosuppression in the treatment of PTLD. With regard to lymphocyte subpopulations, the proportion of HLA-DR+CD8+ lymphocytes was significantly different in non-PTLD controls (33%), responders to PTLD treatment (44%), and nonresponders (70%). There are several possible explanations: This CTL population could be EBV-specific – however, the observation of 80% HLA-DR+CD8+/CD8+ cells in one nonresponder suffering from non-EBV-associated PTLD and low HLA-DR+ frequencies in eight of nine EBV-associated responders (median frequency: 40%) count against this interpretation. A polyclonal expansion of CTLs because of higher levels of pro-inflammatory cytokines in nonresponders is unlikely as pro-inflammatory cytokines such as IL-6 and TNFa are not significantly different in responders and nonresponders at diagnosis. Intriguingly, two studies that monitored the CD8-positive T-cell population in bone marrow transplant recipients who developed PTLD showed that reduction of immunosuppression in these patients was associated with an increase of CD8+ T cells, an expansion of oligoclonal CTL populations and resolution of disease [33,34]. In one patient with EBV-associated PTLD treated with allogenic EBV-specific T cells, the early peak of CTL precursors in peripheral blood following CTL infusions coincided with an increase in HLA-DR+CD8+ T cells (activated cytotoxic T-cell phenotype) in the circulation with reversal of CD4/CD8 ratio [35]. Thus, our observation of a sustained increase of this very T-cell subset in nonresponders to PTLD treatment from diagnosis to the end of follow-up might indicate a futile attempt of the immune system to attack the malignant clone. Although in the end, the specificity of these cells is unclear, their increase might represent a compensatory clonal expansion of functionally compromised tumor-specific CTLs.

Authorship

RUT and PR: designed the study, co-ordinated the research, and take primary responsibility for the paper. CH, SW, DE, RN, NB, and HR: recruited the patients and collected the data. SW, CH, RUT, and PS: analyzed the data. PS: did the statistical modeling. RUT, NB, HR, and PR: interpreted the data. IA: served as the reference pathologist. BG: served as a reference virologist. HZ, CH, and RUT: wrote the paper.

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